Allelic Imbalance, Including Deletion of *PTEN/MMAC1*, at the Cowden Disease Locus on 10q22-23, in Hamartomas From Patients With Cowden Syndrome and Germline *PTEN* Mutation

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Cowden disease (CD) is a rare, autosomal dominant inherited cancer syndrome characterized by multiple benign and malignant lesions in a wide spectrum of tissues. While individuals with CD have an increased risk of breast and thyroid neoplasms, the primary features of CD are hamartomas. The gene for CD has been mapped by linkage analysis to a 6 cM region on the long arm of chromosome 10 at 10q22-23. Loss of heterozygosity (LOH) studies of sporadic follicular thyroid adenomas and carcinomas, both component tumors of CD, have suggested that the putative susceptibility gene for CD is a tumor suppressor gene. Somatic missense and nonsense mutations have recently been identified in breast, prostate, and brain tumor cell lines in a gene encoding a dual specificity phosphatase, *PTEN/MMAC1*, mapped at 10q23.3. Furthermore, germline *PTEN/MMAC1* mutations are associated with CD. In the present study, 20 hamartomas from 11 individuals belonging to ten unrelated families with CD have been examined for LOH of markers flanking and within *PTEN/MMAC1*. Eight of these ten families have germline *PTEN/MMAC1* mutations. LOH involving microsatellite markers within the CD interval, and including *PTEN/MMAC1*, was identified in two fibroadenomas of the breast, a thyroid adenoma, and a pulmonary hamartoma belonging to 3 of 11 (27%) of these patients. The wild-type allele was lost in these hamartomas. Semi-quantitative PCR performed on RNA from hamartomas from three different tissues from a CD patient suggested substantial reduction of *PTEN/MMAC1* RNA levels in all of these tissues. The LOH identified in samples from individuals with CD and the suggestion of allelic loss and reduced transcription in hamartomas from a CD patient provide evidence that *PTEN/MMAC1* functions as a tumor suppressor in CD. *Genes Chromosomes Cancer 21:61–69, 1998.*

INTRODUCTION

Cowden disease (CD), or multiple hamartoma syndrome (MIM 158350), is an infrequently recognized autosomal dominant inherited cancer syndrome with variable expressivity. Clinically, it is characterized by a wide spectrum of benign and malignant lesions involving multiple organ systems. Patients with CD have a particularly high risk of breast and thyroid cancers, but the major features of CD are hamartomas (reviewed in Eng et al., 1994; Longy and Lacombe, 1996; Eng, 1997). Hamartomas are developmentally incorrect, disorganized, hyperplastic non-malignant growths. The pathognomonic "hamartomas" in CD are trichilemmomas of the skin and mucocutaneous papillomatous papules (reviewed in Hanssen and Fryns, 1995; Longy and Lacombe, 1996; Eng, 1997). Such mucocutaneous lesions occur in 99% of individuals with CD. Other

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significant component hamartomas include thyroid adenomas and goiters (40–60% of individuals with CD), fibroadenomas of the breast (approximately 70% of affected females), and hamartomatous polyps of the gastrointestinal tract (35–40%). The two component cancers, those of the breast and thyroid, occur with a frequency of 25–50% and 3–10%, respectively.

The *CD* locus was mapped to a 6 cM interval at 10q22–23 (Nelen et al., 1996). No genetic heterogeneity had been detected in 13 informative CD families (Nelen et al., 1996; Longy et al., unpublished). Allelic imbalance or loss of heterozygosity (LOH) in the *CD* locus had been observed in some sporadic component tumors of CD, such as uterine leiomyomas (Jones et al., 1994) and follicular thyroid tumors (Marsh et al., 1997). Recently, the *CD* gene was identified as *PTEN/MMAC1* encoding a dual specificity phosphatase (Li et al., 1997; Liaw et al., 1997; Steck et al., 1997; Myers et al., 1997). Germline missense and nonsense point mutations were reported in four of five CD families (Liaw et al., 1997). LOH in the *CD* region in sporadic component tumors and nonsense mutations in *PTEN/MMAC1* provide highly suggestive evidence that the *CD* susceptibility gene encodes a tumor suppressor. We examined whether LOH in tumors from individuals with CD and germline *PTEN/MMAC1* mutations involved the wild-type allele, since this would provide further evidence that *PTEN/MMAC1* is a tumor suppressor gene conforming to Knudson's "2-hit" model of tumorigenesis (Knudson et al., 1971). Reduced transcription of this gene in Cowden hamartoma tissue would also suggest a tumor suppressor function, or loss thereof. Therefore, we characterized 11 CD individuals for germline *PTEN/MMAC1* mutation and sought to determine if allelic imbalance was present within *PTEN/MMAC1* and flanking regions in 20 hamartomas from these individuals. In addition, we also explored the level of transcription of this gene in affected tissue from a Cowden patient by semi-quantitative RT-PCR.

MATERIALS AND METHODS

Hamartoma Samples

Twenty hamartoma specimens, including fibroadenomas of the breast (four samples), thyroid adenomas (three), hamartomatous polyps of the gastrointestinal tract (six), trichilemmomas and papillomatous papules of the skin and mucosa (four), and hamartomas of the lung (two) and kidney (one), were collected from 11 individuals belonging to ten distinct families with CD. Two individuals, patients 6 and 11, were affected siblings from the same CD family. The diagnoses of CD were made in accordance with the criteria of the International Cowden Consortium (Nelen et al., 1996; Eng 1997).

DNA and RNA Preparation

Tissue was collected at the time of surgery and snap frozen or formalin-fixed and paraffin-embedded. Blood samples were also collected from these 11 individuals. High molecular weight DNA was obtained from the snap frozen tissue by proteinase K digestion, phenol-chloroform extraction, and ethanol precipitation (Mathew et al., 1987). DNA was obtained from paraffin-embedded tissue using the QIAamp® Tissue Kit (Qiagen, Inc., Chatsworth, CA). Constitutional DNA from each patient was obtained by DNA extraction from blood leukocytes by using standard techniques (Mathew et al., 1987).

RNA was obtained from three hamartomas, thyroid, lung, and kidney from patient 6 using Tri Reagent according to the manufacturer's instructions (Sigma Chemical Co., St Louis, MO). RNA was also obtained from six normal thyroids used as controls.

PTEN/MMAC1 Mutation Analysis

The nine exons of *PTEN/MMAC1* were sequenced by using nested primers designed within flanking intronic sequence. PCR conditions and primers have been previously described (Liaw et al., 1997; Steck et al., 1997) with the exception of primers for exons 2 and 4, respectively, P10X2AF102 (5'-GTT TGA TTG CTG CAT ATT TCA G -3') and P10X2AR88 (5'-TCT AAA TGA AAA CAC AAC ATG-3'), P10X2CF51 (5'-CAT TAT AAA GAT TCA GGC AAT G-3'), and P10X2CR108 (5'-GAC AGT AAG ATA CAG TCT ATC-3'). PCR products were gel isolated and purified by using the Wizard®PCR Preps DNA Purification System (Promega, Madison, WI). Direct sequencing of these products was performed by using the ABI Prism Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer Corp., Norwalk, CT). Cycle sequencing products were electrophoresed on 6% Long Ranger gels (FMC Bioproducts, Rockland, ME) and analyzed on an Applied Biosystems model 373A automated DNA sequencer (Perkin-Elmer Corp., Norwalk, CT).

Detection of LOH and *PTEN/MMAC1* Deletion

The *CD* gene had been previously mapped to a 5 cM region at 10q22-23 defined by the marker loci

D10S215 and *D10S564* (Gyapay et al., 1994; Nelen et al., 1996). However, recent mapping has redefined this region to span a distance of at least 6 cM (Dib et al., 1996). Twelve polymorphic markers within and flanking the *CD* interval were employed for the detection of LOH: *D10S219* (AFM240wg7); *D10S1786* (AFMa070xe9); *D10S551* (AFM240vf10); *D10S1644* (AFMa124wd9); *D10S1744* (AFM063xb10); *D10S579* (AFM282yc1); AFMa086wg9; *PTEN* IVS8 +32T/G, *D10S541* (AFM205xe3); *D10S1739* (AFMb362yg5); *D10S564* (AFM029xh12); and *D10S583* (AFM289zh5) (Dib et al., 1996). The markers are ordered from centromeric to telomeric and reflect the integrated genetic and physical map of chromosome 10q (http://www-genome.wi.mit..edu). AFMa086wg9 is located within intron 2 of *PTEN/MMAC1*, thus placing the *CD* gene between the markers *D10S579* and *D10S541*. The T/G sequence polymorphism lies within intron 8 of *PTEN*. Heterozygosity exists for approximately 40% of individuals. This T/G polymorphism was distinguished by *Hin*cII (New England Biolabs, Beverly, MA) restriction digestion of the exon 8 PCR product. A second *Hin*cII site within the PCR fragment served as an internal control. *D10S541* was used instead of *D10S215* (AFM205wd12) because the latter resulted in erratic PCR amplification and contained null alleles (Marsh et al., 1997). Further, D10S215 and *D10S579* are a few hundred kilobases from each other, although it is unclear which is centromeric (Li et al., 1997).

PCR products for microsatellite analysis were generated using a Touchdown Thermal Cycler (Hybaid Limited, Middlesex, UK) and the following protocol: 10 cycles of denaturation at 94°C for 1 minute, annealing at 70°C for 1 minute (decreasing 1°C each cycle until reaching a temperature of 61°C), and extension at 72°C for 1 minute; 25 cycles of 94°C for 40 seconds, annealing at 55°C for 1 minute and extension at 72°C for 2 minutes; and followed by 72°C for 10 minutes. The final concentrations of PCR reagents were as follows: 1 μM of each primer, 0.125 mM of each deoxyribonucleotide triphosphates, 1× PCR buffer containing 1.5 mM $MgCl_2$ (Perkin Elmer Cetus, Norwalk, CT) and 0.3 μl *Taq* polymerase (5 units/μl) (Perkin Elmer Cetus, Norwalk, CT). PCR reactions were performed in a final volume of 22 μl.

Each forward primer defining each microsatellite repeat marker was 5'-tagged with the fluorescent dye labels HEX or 6-FAM (Genosys Biotechnologies, Inc., TX). PCR products were electrophoresed on 6% denaturing polyacrylamide gels by using an Applied Biosystems model 373A automated DNA sequencer (Applied Biosystems, Perkin Elmer Cetus, Norwalk, CT) and the Genescan -2500 ROX internal size standard (Genescan, Applied Biosystems, Foster City, CA). The results were analyzed by automated fluorescence detection by using Genescan 672 collection and analysis software (Genescan, Applied Biosystems, Foster City, CA). Scoring of LOH was performed by inspection of the Genescan analysis output. A double peak observed for the microsatellite marker amplified from DNA extracted from the blood sample indicated a heterozygote. A single peak in DNA extracted from the corresponding hamartoma tissue indicated loss of one allele. If normal cells were mixed with tumor cells, a minimum ratio of 1.5:1 of germline DNA peak to tumor DNA peak was considered an indication of LOH.

PEN/MMAC1 Dosage and Expression Analysis

In order to control for normal tissue contamination of hamartoma samples, a genomic-based semi-quantitative duplex PCR was performed on germline and hamartoma DNA from individual 6 by using primers previously described for exon 8 of *PTEN/MMAC1* (Liaw et al., 1997) and those for an unrelated, single copy gene, *KIP1/P27* (P27F 5'-TTG CCC GAGTTC TAC TAC AGA-3' and P27R 5'-TTA CGT TTG ACG TCT TCT GAG-3') under the conditions described above with the exception of a reduced cycle number of cycles (n=28) in order to ensure that the PCR reaction remained in the exponential phase of the logarithmic synthesis curve at the time of analysis. The relative amounts of the *PTEN/MMAC1* PCR fragment versus that of *KIP1/P27* were determined by visual inspection and densitometric scanning using ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

A second semi-quantitative assay was developed to assess *PTEN/MMAC1* transcription in hamartoma tissues. RNA from hamartoma tissue was reverse transcribed into cDNA by using random hexamers and avian myeloblastosis virus (AMV) reverse transcriptase (Promega, Madison, WI) following the manufacturer's instructions. Fifty nanograms of the cDNA product was used as template in a duplex PCR reaction using exonic primers for *PTEN/MMAC1* spanning exons 1 to 5 (F 5'-CAT CTC TCT CCT CCT TTT TCT TCA-3' and R 5'-TTG TGC AAC TCT GCA ATT AA-3') and previously published primers for the single copy house-keeping gene glyceraldehyde-phosphate dehydrogenase (*GAPDH*) (Dahia et al., 1996). The PCR conditions were as described above with a

TABLE 1. Germline *PTEN* mutations in Cowden Patients and Somatic Deletions in Their Hamartomas

Individual	Germline *PTEN* mutation	Hamartoma type	*PTEN* LOH (AFMa086wg9)	*PTEN* LOH (IV58 +32T/G)
1	IVS2-2A → G	breast	not informative	not informative
2	Q157X	breast	LOH	not done
3	none detected	breast	not informative	LOH
4	none detected	breast	not informative	not informative
5	R233X	thyroid	retention of heterozygosity	retention of heterozygosity
		corpus ileum	retention of heterozygosity	retention of heterozygosity
6	G129E	thyroid	LOH	LOH
		intestine	retention of heterozygosity	retention of heterozygosity
		kidney	retention of heterozygosity	retention of heterozygosity
		lung	LOH	LOH
7	R130X	thyroid	not informative	not informative
8	R130X	skin	not informative	not informative
		gingiva	not informative	not informative
		lung	not informative	not informative
9	R130L	colon	not informative	retention of heterozygosity
		oral	not informative	retention of heterozygosity
10	C124R	colon	not informative	not informative
		rectum	not informative	not informative
11	G129E	skin	not informative	not informative

reduced cycle number (n=28). Again, PCR product ratios were obtained by visual inspection and densitometric scanning.

Haplotype Generation

In order to assess which allele, the *CD* allele or the wild-type allele, was lost in tumors where LOH was observed, haplotypes of the individual's family, where possible, were generated using eight dinucleotide repeat microsatellite markers as described above.

RESULTS

Germline *PTEN/MMAC1* Analysis in CD Patients

Germline *PTEN/MMAC1* mutations were found in eight of ten unrelated CD families (Table 1). The two families in which a mutation was not identified did not have unusual clinical features. Of the eight mutations identified, three were missense point mutations (not seen in 100 normal alleles sequenced), four were nonsense point mutations, and one was a splice site mutation (IVS2 -2A→G). While individuals 7 and 8 were found to have the identical germline nonsense mutation R130X, these mutations did not arise on identical 10q22–23 haplotypes (data not shown).

LOH and *PTEN* Deletion Identified in Hamartomas From Patients With CD

DNA from 20 hamartoma specimens from 11 individuals belonging to ten families with CD were screened for LOH using 12 polymorphic markers in a 20 cM interval defined by and including *D10S219* and *D10S583* (Fig. 1). All individuals were informative for a minimum of three markers, individual 1 being informative for 9 of the 12 markers studied. LOH was detected in four hamartomas from three CD individuals (patients 2, 3, and 6). LOH of markers within *PTEN/MMAC1*, namely, AFMa086wg9 and IVS8 +32G/T, was evident (Figs. 1–4). Of note, *D10S579* and *D10S541*, which flank *PTEN/MMAC1*, retained heterozygosity, while LOH within *PTEN/MMAC1* was observed in a breast fibroadenoma from patient 2 (Fig. 1). This is highly suggestive that specific *PTEN/MMAC1* loss represents the second genetic event or "hit" in these hamartomas.

A second deletion unit, telomeric of *D10S541* (patient 2) and centromeric of *D10S564* (patient 6, thyroid), is also present. Germline homozygosity at *D10S541* in patient 6 did not allow us to determine whether loss of one 10q23 allele in this patient's tissues could encompass both *PTEN/MMAC1* and possibly other gene(s) within this second region as well.

PTEN/MMAC1 allele loss in hamartomas from patient 6 were clearly observed in two of the four tissues examined by polymorphic marker analysis. We therefore believed that the apparent retention of heterozygosity in this patient's kidney and intestine hamartomas could reflect normal tissue contamination. Thus, we sought to show the existence of specific *PTEN/MMAC1* allele loss in all four

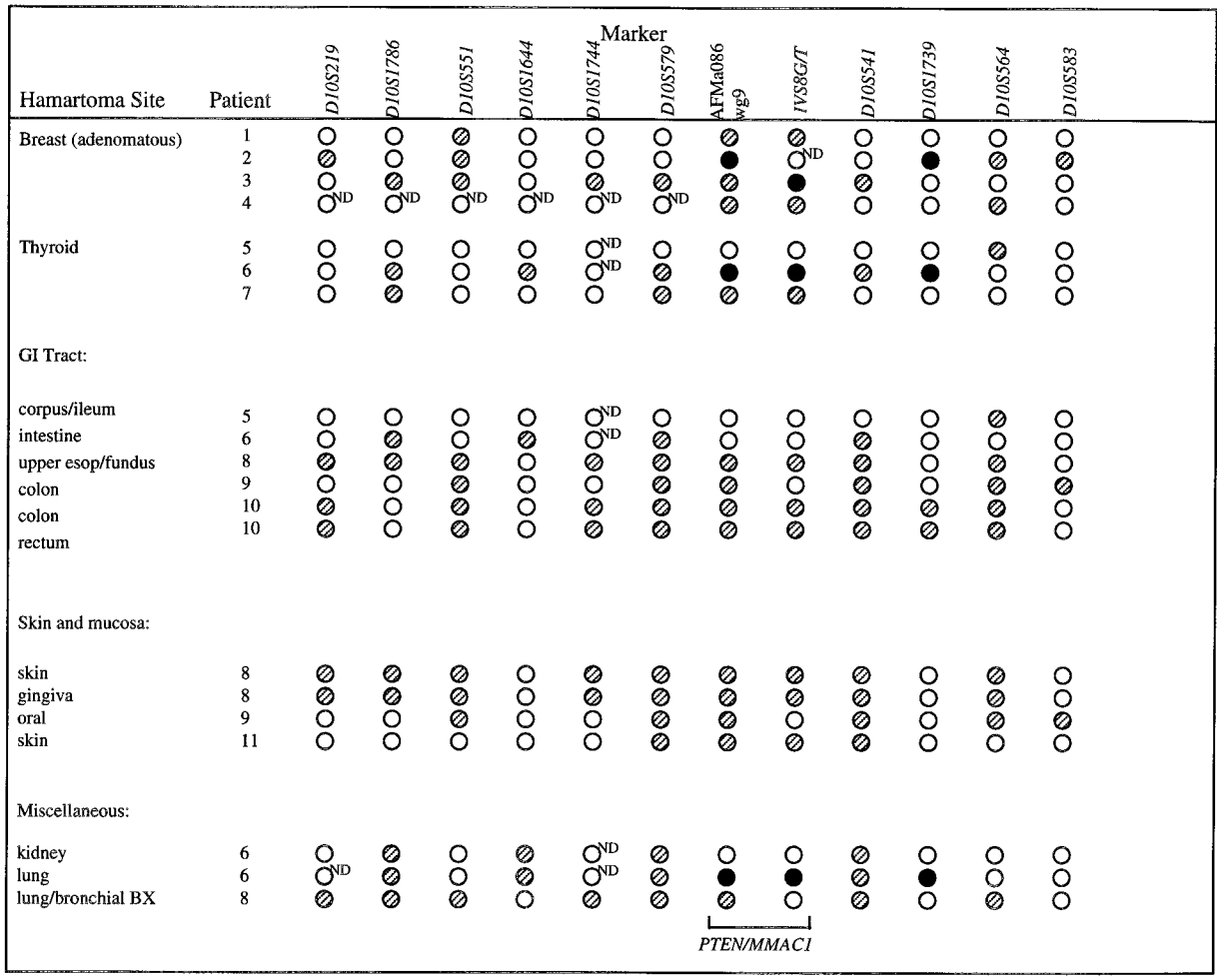

Figure 1. LOH analysis of 12 polymorphic markers within 10q22–24 in 20 hamartomas from 11 individuals belonging to ten unrelated CD families. Clear circles represent retention of heterozygosity, hatched circles homozygosity (not informative), and solid circles LOH. "ND" indicates that this sample has not been analyzed at a specific locus due to technical difficulties. The position of *PTEN/MMAC1* is indicated.

hamartomas by a genomic DNA-based semi-quantitative duplex PCR by using primers to amplify the unrelated, single copy gene *KIP1/P27* (Fig. 3). Visual inspection and densitometric scanning of the gel were highly suggestive of LOH in all four hamartoma tissues (Table 1, Fig. 3).

To further demonstrate that deletion of a *PTEN* allele translates into decreased RNA levels, a semi-quantitative RT-PCR assay was designed to assess *PTEN/MMAC1* transcription in three hamartoma tissues (lung, thyroid, and kidney) from patient 6. In all cases, the hamartoma showed decreased *PTEN/MMAC1* transcript levels when compared to the unrelated single copy gene *GADPH*, thus confirming the genomic DNA deletion data (Fig. 5).

Haplotype Analysis

In order to determine whether the observed LOH always correlated with loss of the allele containing the mutated *CD* gene or that containing the wild-type *CD* locus, haplotypes were generated for individuals 6 and 11 (affected individuals from the same family) and individual 2 and her immediate family members using eight microsatellite markers, which were included in the LOH studies. In all three hamartomas where LOH was observed, the wild-type alleles were consistently lost (Fig. 6).

DISCUSSION

Germline *PTEN/MMAC1* mutations were identified in eight of ten CD families, including three missense and four nonsense point mutations as well as a single splice site mutation. It is possible that the two families without *PTEN/MMAC1* mutation may have a mutation in the promoter region of this gene or a large germline deletion of the entire gene, although the heterozygous nature of the IVS8 polymorphism in the germline of patient 3 would,

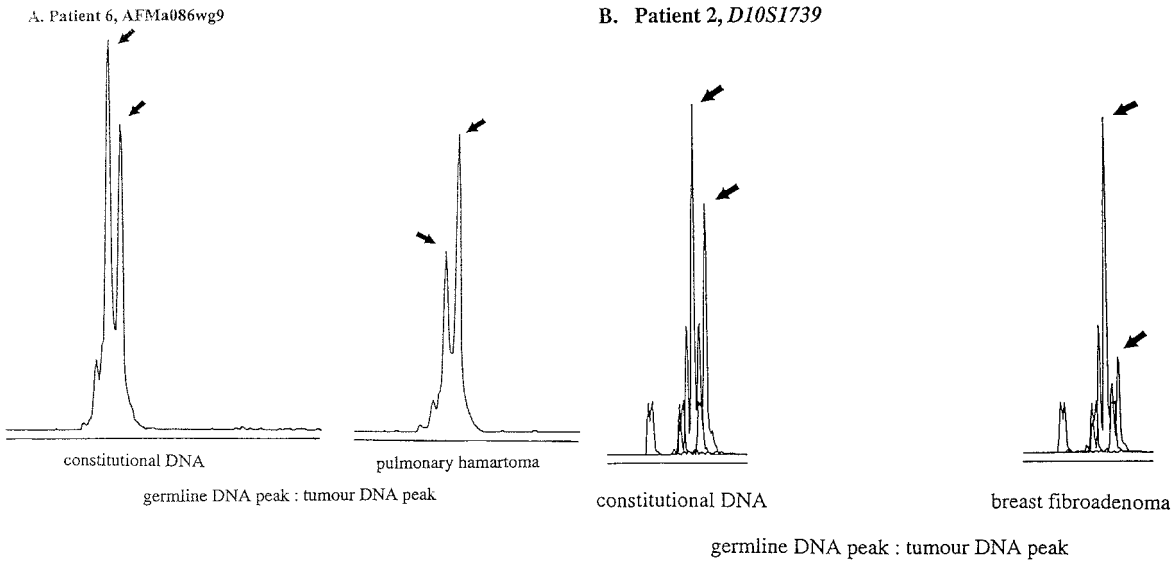

Figure 2. Representative Genescan analysis outputs showing LOH in DNA from blood-hamartoma pairs at AFMa086wg9 (within *PTEN/MMAC1*) and *D10S1739*. **A:** LOH at AFMa086wg9 in the pulmonary hamartoma from patient 6. **B:** LOH at *D10S1739* in the breast fibroadenoma from patient 2. In all cases, a minimum ratio of 1.5:1 of germline DNA peak to tumor DNA peak was considered an indication of LOH.

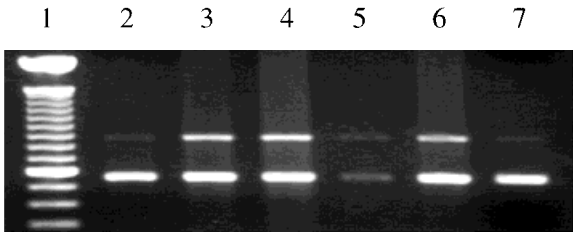

Figure 3. Semi-quantitative duplex PCR showing amplification of *PTEN/MMAC1* exon 8, approximately 550 bp, and the unrelated, single-copy gene *KIP1/P27*, 948 bp, in CD hamartomas from patient 6. **Lane 1**, 100 bp DNA ladder (Gibco BRL Life Technologies, Gaithersburg, MD); **lane 2**, constitutive DNA from patient 6; **lane 3**, thyroid hamartoma; **lane 4**, intestinal polyp; **lane 5**, kidney hamartoma; **lane 6**, pulmonary hamartoma; and **lane 7** control DNA (Promega, Madison, WI). Visual inspection and densitometric scanning gave results consistent with a reduction of the level of genomic *PTEN/MMAC1* in hamartoma tissue relative to constitutive DNA compared to the *KIP1/P27* control fragment.

at least in this patient, exclude the latter. Unfortunately, the microsatellite marker AFMa086wg9 was homozygous, and thus non-informative, in these three individuals. No significant genotype/phenotype correlations could be drawn from this small group of patients. Such a correlation would require mutation analysis of more CD families.

Two separate regions of allelic imbalance of markers flanking and including *PTEN/MMAC1* were detected by using polymorphic markers in hamartoma tissue from 27% of the CD individuals studied. In each of the three hamartomas (breast fibroadenoma, thyroid adenoma, and pulmonary hamartoma) in which definite LOH was observed, the wild-type allele was lost. Allelic deletion of *PTEN/MMAC1* was indicated in a fourth hamartoma, a breast fibroadenoma from individual 3, by differential digestion at the *Hin*cII polymorphic site (Fig. 4). This individual was non-informative for the markers flanking this polymorphism, including the marker within intron 2 of *PTEN/MMAC1*, AFMa086wg9, thus making it impossible to assess whether this LOH was limited to *PTEN/MMAC1* or was more extensive. Further attempts to analyze this sample in a semi-quantitative duplex PCR did not yield results given that this tissue sample was archival and not amenable to the amplification of fragments over 300 bp, while remaining in the exponential phase of the amplification curve for duplexed primers.

Retention of the mutant allele in hamartoma samples from CD patients, coupled with somatic loss of the wild-type allele within the same region, provides evidence that *PTEN/MMAC1* encodes a tumor suppressor and that mutations within *PTEN/MMAC1* are etiologic for CD and its tumors, even benign ones. This was clearly shown to be the case in at least three patients with germline *PTEN/MMAC1* mutation, individuals 2, 3, and 6. Thus, it would appear that tumorigenesis in CD adheres to Knudson's two-mutation model (Knudson, 1971). According to this model, the first mutation in a tumor suppressor gene occurs in the germline in the instances where an inherited cancer syndrome is involved. Subsequently, a second (somatic) muta-

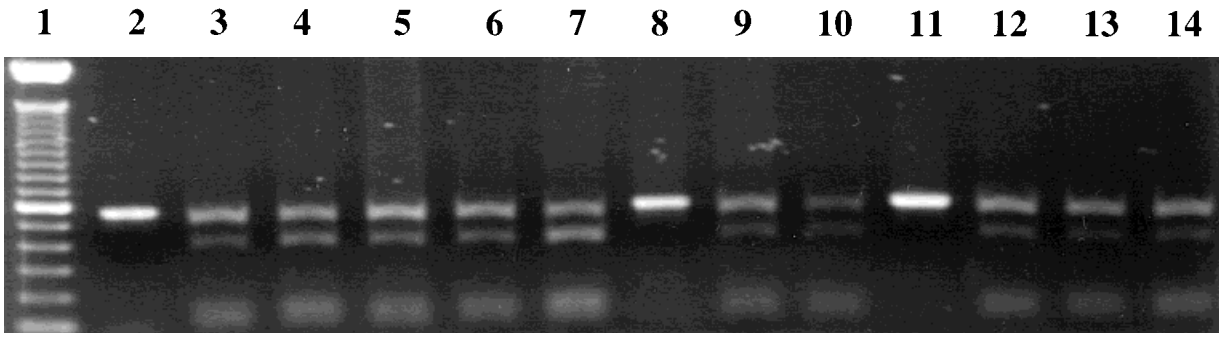

Figure 4. Differential digestion with *Hin*cII at the IVS8 +32 T/G polymorphic site in constitutive DNA/hamartoma pairs from CD patients. **Lane 1**, 100 bp DNA ladder (Gibco BRL Life Technologies, Gaithersburg, MD); **lane 2**, uncut constitutive DNA from patient 6; **lane 3**, constitutive DNA from patient 6 digested with *Hin*cII; **lane 4**, thyroid hamartoma DNA from patient 6 digested with *Hin*cII; **lane 5**, intestinal polyp DNA from patient 6 digested with *Hin*cII; **lane 6**, kidney hamartoma DNA from patient 6 digested with *Hin*cII; **lane 7**, pulmonary hamartoma DNA from patient 6 digested with *Hin*cII; **lane 8**, uncut constitutive DNA from patient 3; **lane 9**, constitutive DNA from patient 3 digested with *Hin*cII; **lane 10**, DNA from breast fibroadenoma of patient 3 digested with *Hin*cII; **lane 11**, uncut constitutive DNA from patient 9; **lane 12**, constitutive DNA from patient 9 digested with *Hin*cII; **lane 13**, colon hamartoma from patient 9 digested with *Hin*cII; and **lane 14**, oral mucosal hamartoma from patient 9 digested with *Hin*cII. DNA from hamartomas belonging to patients 3 and 6 show a different digestion fragment intensity pattern compared to their paired blood samples, suggestive of LOH perhaps with normal tissue contamination. DNA from the blood/hamartoma pairs of patient 9 showed a similar digestion intensity pattern, thus demonstrating no LOH.

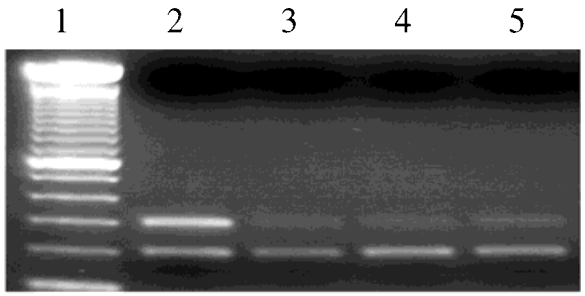

Figure 5. Semi-quantitative RT-PCR assay showing decreased *PTEN/MMAC1* RNA levels in CD hamartomas from patient 6 in comparison to normal thyroid tissue and a single copy house-keeping gene glyceraldehyde-phosphate dehydrogenase (GAPDH). **Lane 1**, 100 bp DNA ladder (Gibco BRL Life Technologies, Gaithersburg, MD); **lane 2**, normal thyroid tissue; **lane 3**, thyroid hamartoma; **lane 4**, pulmonary hamartoma; **lane 5**, kidney hamartoma. Densitometric scanning gave the following relative ratios for *PTEN/MMAC1* expression compared to that of *GAPDH*: normal thyroid, 2.63; thyroid hamartoma, 0.38; pulmonary hamartoma, 0.21; and kidney hamartoma, 0.29. These ratios represent a 7–12-fold decrease in *PTEN/MMAC1* RNA levels in Cowden hamartomas compared to normal thyroid tissue.

tion arising on the opposite allele causes complete loss of function of the tumor suppressor in a given cell and leads to tumor formation. This model has been shown to be robust in a number of familial cancer syndromes including retinoblastoma (*RB1*) (Kato et al., 1994), familial adenomatous polyposis coli (*APC*) (Ichii et al., 1992), neurofibromatosis type 2 (*NF2*) (Irving et al., 1994), and Wilm's tumor (*WT1*) (Tadokoro et al., 1992; Gessler et al., 1993). Preliminary studies in three hamartomas from a single CD patient suggest reduced expression of *PTEN/MMAC1*, consistent with a tumor suppressor function for this gene.

The current study examines LOH in CD hamartomas and not carcinomas. Interestingly, in sporadic brain tumor cell lines, somatic mutation in this gene seems to be found in cell lines derived from more advanced cancers (Steck et al., 1997). Yet, our data show that a reduction of *PTEN/MMAC1* transcription (germline mutation and deletion of remaining allele) can cause hamartoma formation, at least in a proportion of cases. The apparent lack of LOH in the region of the *CD* locus in the majority of CD patients in this study has several explanations. First, detection of LOH may have been confounded by a lack of informative markers in this region. Second, there may be no second allele loss but instead small mutations representing the second hit. Third, in the remaining hamartomas, gene silencing may be achieved through other mechanisms such as methylation or transcript destabilization. Finally, it may be argued that the germline mutation in *PTEN/MMAC1* is critical for hamartoma formation but that the second event represented by LOH would invariably lead to carcinoma formation; hence, a higher frequency of LOH would be detected in carcinomas from CD patients. However, a breast adenocarcinoma from a CD individual has shown no LOH in the critical interval (Longy et al., unpublished). Unfortunately, no germline *PTEN/MMAC1* mutation could be detected in this patient (Longy, unpublished).

The function and consequences of mutation of *PTEN/MMAC1* are open to speculation. Taking together the clinical epidemiology of CD, i.e., hamartomas in almost 100% of CD individuals and cancers in a minority, the germline and LOH data in the present study, as well as preliminary expression data, we have strong evidence that *PTEN/*

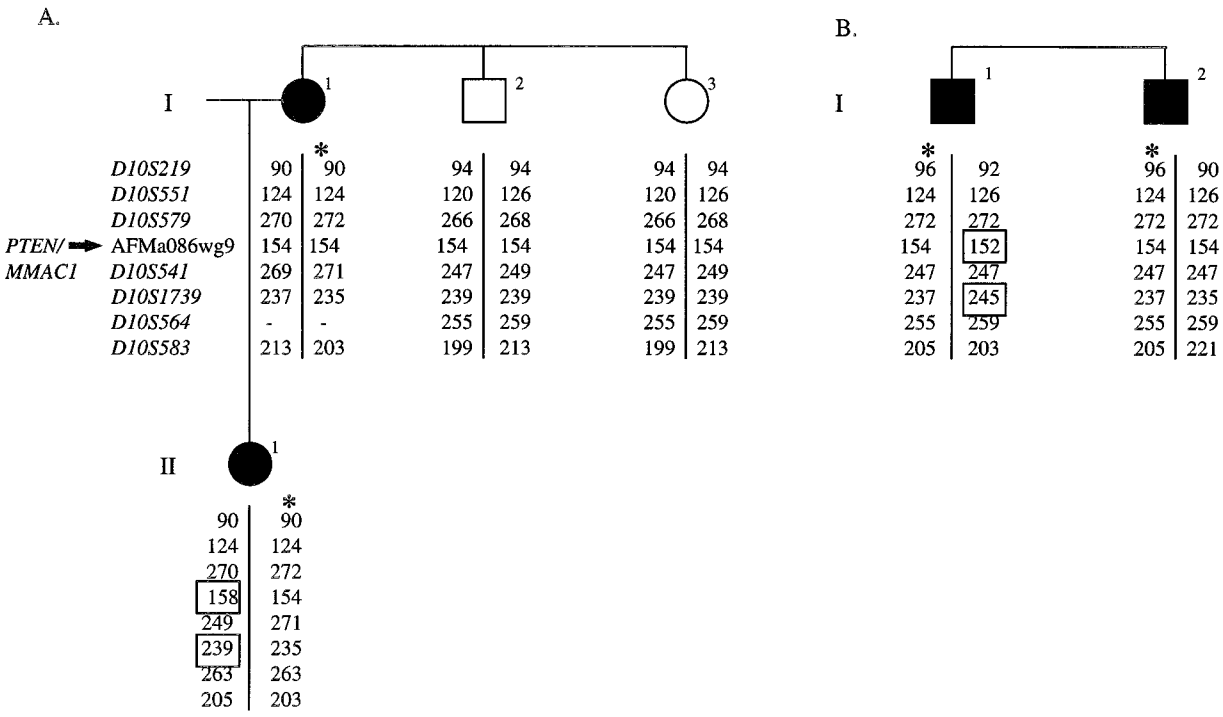

Figure 6. Partial pedigrees and haplotypes of CD patients showing loss of the wild-type allele. **A:** Partial pedigree and haplotype of patient 2 (II-1). LOH in this patient's breast fibroadenoma is indicated on the wild-type allele at AFMa086wg9 and *D10S1739*. Clear haplotype sharing of the disease allele can be observed between this patient and her affected mother, *, but not the mother's unaffected siblings. *D10S564* was unable to be amplified by PCR for I-1. **B:** Partial pedigree showing affected siblings, patient 6 (I-1) and patient 11 (I-2). LOH in the thyroid adenoma and pulmonary hamartoma of patient 6 is indicated on the wild-type allele at AFMa086wg9 and *D10S1739*. Haplotype sharing, presumably of the disease allele, *, is observed between these two affected siblings.

MMAC1 functions as a tumor suppressor in CD. Loss of function of *PTEN/MMAC1* may lead to hamartomas and a proliferative advantage. The formation of carcinoma might be favored on such a proliferative background but other genetic and/or epigenetic events may also be necessary for malignant transformation.

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REFERENCES

Dahia PLM, Ahmed-Shuaib A, Jacobs RA, Chew SL, Honegger J, Fahlbusch R, Besser GM, Grossman AB (1996) Vasopressinreceptor expression and mutation analysis in corticotropin-secreting tumors. J Clin Endocrinol Metab 81:1768–1771.

Dib C, Faure S, Fizames C, Samson D, Drouot N, Vignal A, Millasseau P, Marc S, Hazan J, Seboun E, Lathrop M, Gyapay G, Morissette J, Weissenbach J (1996) A comprehensive genetic map of the human genome based on 5,264 microsatellites. Nature 380:152–154.

DuVillard JA, Schlumberger M, Wicker R, Caillou B, Rochefort P, Feunteun J, Monier R, Parmentier C, Suarez HG (1995) Role of *ras* and *gsp* oncogenes in human epithelial thyroid tumorigenesis J Endocrinol Invest 18:124–126.

Eng C. (1997) Cowden syndrome. J Genet Counsel 6:181–191.

Eng C, Murday V, Seal S, Mohammed S, Hodgson SV, Chaudary MA, Fentiman IS, Ponder BA, Eeles RA. (1994) Cowden syndrome and Lhermitte-Duclos disease in a family: A single genetic syndrome with pleiotropy? J Med Genet 31:458–461.

Fagin JA (1994) Molecular genetics of human thyroid neoplasms. Annu Rev Med 45:45–52.

Gessler M, Konig A, Moore J, Qualman S, Arden K, Cavenee W, Bruns G (1993) Homozygous inactivation of WT1 in a Wilms' tumor associated with the WAGR syndrome. Genes Chromosomes Cancer 7:131–136.

Gyapay G, Morissette J, Vignal A, Dib C, Fizames C, Millasseau P, Marc S, Bernardi G, Lathrop M, Weissenbach J. (1994) The 1993–94 Généthon human genetic linkage map. Nature Genet 7:246–339.

Hanssen AMN, Fryns JP (1995) Cowden syndrome. J Med Genet 32:117–119.

Ichii S, Horii A, Nakatsuru S, Furuyama J, Utsunomiya J, Nakamura Y (1992) Inactivation of both APC alleles in an early stage of colon adenomas in a patient with familial adenomatous polyposis (FAP) Hum Mol Genet 1:387–390.

Irving RM, Moffat DA, Hardy DG, Barton DE, Xuereb JH, Maher ER. (1994) Somatic NF2 gene mutations in familial and non-familial vestibular schwannoma. Hum Mol Genet 3:347–350.

Jones MH, Koi S, Fujimoto I, Hasumi K, Kato K, Nakamura Y (1994) Allelotype of uterine cancer by analysis of RFLP and microsatellite polymorphisms: Frequent loss of heterozygosity on chromosome arms 3p, 9q, 10q, and 17p. Genes Chromosomes Cancer 9:119–123.

Kato MV, Ishizaki K, Shimizu T, Ejima Y, Tanooka H, Takayama J, Kaneko A, Toguchida J, Sasaki MS (1994) Parental origin of

germ-line and somatic mutations in the retinoblastoma gene. Hum Genet 94:31–38.

Knudson AG (1971) Mutation and cancer: Statistical study of retinoblastoma. Proc Natl Acad Sci USA 68:820–823.

Li J, Yen C, Liaw D, Podsypanina K, Bose S, Wang S, Puc J, Miliaresis C, Rodgers L, McCombie R, Bigner SH, Giovanella BC, Ittman M, Tyoko B, Hibshoosh H, Wigler MH, Parsons R (1997) *PTEN*, a putative protein tyrosine phosphatase gene mutated in human brain, breast and prostate cancer. Science 275:1943–1947.

Liaw D, Marsh DJ, Li J, Dahia PLM, Wang SI, Zheng Z, Bose S, Call KM, Tsou HC, Peacocke M, Eng C, Parsons R (1997) Germline mutations of the PTEN gene in Cowden disease, an inherited breast and thyroid cancer syndrome. Nature Genet 16:64–67.

Longy M, Lacombe D (1996) Cowden disease: Report of a family and review. Ann Genet 39:35–42.

Marsh DJ, Zheng Z, Zedenius J, Kremer H, Padberg GW, Larsson C, Longy M, Eng C (1997) Differential loss of heterozygosity in the region of the Cowden locus within 10q22-23 in follicular thyroid adenomas and carcinomas. Cancer Res 57:500–503.

Mathew CG, Smith BA, Thorpe K, Wong Z, Royle NJ, Jeffreys AJ, Ponder BAJ (1987) Deletion of genes on chromosome 1 in endocrine neoplasia. Nature 328:524–526.

Myers MP, Stolarov JP, Eng C, Li J, Wang SI, Wigler MH, Parsons R, Tonks NK (1997) P-TEN, the tumor suppressor from human chromosome 10q23, is a dual specificity phosphatase. Proc Natl Acad Sci USA 94:9052-9057.

Nelen MR, Padberg GW, Peeters EAJ, Lin AY, van den Helm B, Frants RR, Coulon V, Goldstein AM, van Reen MMM, Easton DF, Eeles, RA, Hodgson S, Mulvihill JJ, Murday VA, Tucker MA, Mariman ECM, Starink TM, Ponder BAJ, Ropers HH, Kremer H, Longy M, Eng C. (1996) Localization of the gene for Cowden disease to chromosome 10q22-23. Nature Genet 13:114–116.

Pierotti MA, Bongarzone I, Borrello MG, Greco A, Pilotti S, Sozzi G. (1996) Cytogenetics and molecular genetics of carcinomas arising from thyroid epithelial follicular cells. Genes Chromosomes Cancer. 16:1–14.

Steck PA, Pershouse MA, Jasser SA, Slfred Yung WK, Lin H, Ligon AH, Langford LA, Baumgard ML, Hattier T, Davis T, Frye C, Hu R, Swedlund B, Teng DHF, Tavtigian SV (1997) Identification of a candidate tumor suppressor gene, *MMAC1*, at chromosome 10q23.3 that is mutated in multiple advanced cancers. Nature Genet 15:356–362.

Tadokoro K, Fujii H, Ohshima A, Kakizawa Y, Shimizu K, Sakai A, Sumiyoshi K, Inoue T, Hayashi Y, Yamada M (1992) Intragenic homozygous deletion of the *WT1* gene in Wilms' tumor. Oncogene 7:1215–1221.